Cleaning to Achieve Sterility

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Abstract—NASA Planetary Protection regulations state that a surface may be considered "sterile" if a microbial burden of less than 300 aerobic bacterial spores per square meter can be further treated to achieve a 10^4 fold reduction in viable endospores (spores). The results of previous studies [1,2] have suggested that surfaces might be cleaned to a level that is essentially sterile. Here, we report the results of a comparative analysis of the efficacy/ability of three different cleaning approaches to remove bacterial spores from a series of surrogate spacecraft surfaces. In order to accomplish the most realistic and reproducible spore deposition, an aerosol chamber capable of nebulizing innocuous *Bacillus atrophaeus (ATCC, 9372* [formerly *B. subtilis* var niger]) spores was developed and used. This enabled the relatively uniform inoculation of spores as individual entities at a concentration in excess of 10^5 spores per 2.2 cm^2 surrogate surface coupon. Coupons prepared in this fashion were subsequently delivered to three cleaning facilities: Vendor A for cleaning via standard aerospace precision cleaning protocols, Vendor B for cleaning via ultra-pure water, and Vendor C for cleaning via liquid boundary layer disruption. Variations in the chemistry of the cleaning solutions were also explored. Preliminary results suggest that "sterility," as defined by NASA, may indeed be achieved by various cleaning procedures, despite the fact that none of these methods were originally designed, nor are they currently conducted, with such a goal in mind.

TABLE OF CONTENTS

1. INTRODUCTION.....1
2. METHODS 1
3. RESULTS/DISCUSSION.....4
4. CONCLUSIONS 5
5. REFERENCES 7
6. BIOGRAPHY 7
7. ACKNOWLEDGEMENTS 8

1. INTRODUCTION

Since the inception of NASA's planetary exploration program and the dry heat sterilization of the NASA/JPL Viking Landers that first probed the surface of Mars, NASA has mandated that bioburden (endospore forming bacteria) levels on or embedded within the surfaces of electronic and composite structures of spacecraft intended for landing on celestial bodies be limited to predetermined levels that minimize the possibility of contamination. To date, the most effective way of accomplishing this involves dry heat microbial reduction techniques that require spacecraft components to undergo heating at controlled humidity levels for specified time periods. In addition to being very expensive, such strategies are often incompatible with spacecraft materials, and are met with great refute from the spacecraft materials and engineering community.

While the biological definition of sterility is the complete absence of life, NASA deems any surface that undergoes a four-log (10^4) reduction in the number of viable microorganisms (colony forming units, cfu) "sterile."

Consequently, if a cleaning process or processes were unveiled that effectively reduced the number of bacterial spores by the required 10^4 level without the expense and possible detrimental effects to the spacecraft hardware, then the process of readying the spacecraft for final assembly and launch would be drastically simplified.

2. METHODS

The technical approach involved in the evaluation of advanced cleaning methods for this project embodied four central aspects: (1) selection of test materials and the manufacturing and pre-cleaning of test coupons, (2) development of a reproducible spore deposition method, (3) application of suitable cleaning methods, and (4) evaluation of cleaning results.

1

[1] 0-7803-9546-8/06/$20.00© 2006 IEEE
[2] IEEEAC paper #1503, Version 2, Updated Dec. 9, 2005

2.1 Test material selection

Eleven spacecraft materials were selected (Table 1) and cut into 1 x 2.2 cm^2 coupons for use in the following procedures: Bare aluminum, Clean anodized aluminum, Black anodized aluminum, Chemfilmed aluminum, Black epoxy painted aluminum, White painted aluminum, Stainless steel, AstroQuartz I, Graphite composite, Kapton, and Titanium.

2.2 Preparation of material coupons for deposition

Coupons were cleaned in a Freon solution, subjected to UV_{254} light to ensure sterility prior to deposition, and bagged in sterile Mylar bags. One hundred seventy seven coupons of each material type were prepared for analysis.

2.3 Spore deposition methodology

An aerosol spore deposition chamber was constructed to inoculate test coupons. The unit consisted of a 20-liter acrylic chamber that rested on an aluminum base plate. Spore nebulization was accomplished by passing a dry nitrogen stream through a commercially available nebulizer glassware unit whose effluent end was connected to a glass drying-column. From the drying column the gas flow, with nebulized spores, passed through the underside of the base plate into a vertical aluminum deposition tube (Figure 3) on which was mounted three 500 μC ^{210} Po antistatic strips. The spores (singly and in multiples within microdroplets of water) then settled onto the material coupons on the floor of the deposition chamber. A schematic diagram of the deposition process is shown in Figure 1, while Figures 2–4 show the deposition system components.

Primary and secondary regulators were used to control the dry nitrogen propellant that was used for the nebulizer system. Typically this gas flow was set at 25 psig for both

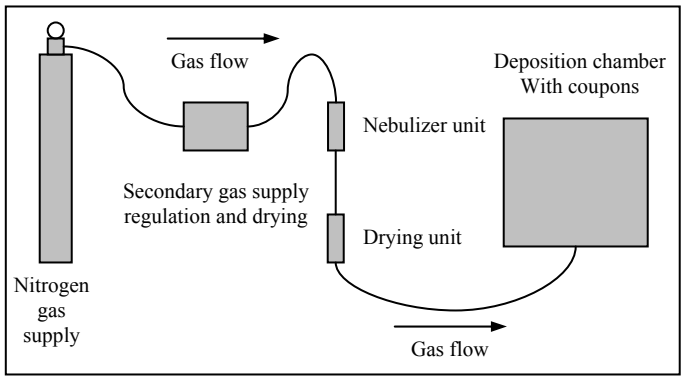

Figure 1. Schematic drawing of spore deposition system

regulators. Individual rotameters (Figure 2) were used to set gas flow rates to the nebulizer and drying tubes. Nitrogen flow was set at 3663 mL/min and 4720 mL/min for the nebulizer and drying column, respectively.

To reduce electrostatic charge build up within the system, the acrylic chamber was sprayed with an electrostatic charge dissipating coating. During operation, the acrylic chamber was grounded to the aluminum base plate, which was in turn, grounded to the building electrical system ground.

A suspension of *Bacillus atrophaeus* purified endospores (10 mL, 10^8 cfu/mL) in sterile water served as inoculae for the system. Aerosol generation time was typically 53 minutes. In order to assure maximum settling of the spores, an 18-20 hour time period was allowed.

Although the probability of accidental spore release was very small, the deposition chamber was contained in a Baker Class II/B3 biosafety cabinet.

Table 1. Baseline Aerosol Deposition on Coupons

Material	Inoculated Count (cfu)		
	Mean	SD	CV (%)
Aluminum, bare (AL)	2.03E+05	4.17E+04	20.54%
Aluminum, black anodized (BA)	1.15E+05	1.16E+04	10.09%
Aluminum, black epoxy paint (BP)	1.48E+05	2.13E+04	14.39%
Aluminum, chemfilmed (CF)	7.13E+04	3.01E+04	42.22%
Aluminum,clear anodized (AC)	9.38E+04	1.48E+04	15.78%
Aluminum, white paint (WP)	1.84E+05	4.56E+04	24.78%
AstroQuartz I (AQ)	1.19E+05	2.18E+05	183.19%
Graphite composite (GC)	2.03E+05	9.05E+04	44.58%
Kapton (K)	3.48E+05	1.36E+05	39.08%
Stainless steel (SS)	2.45E+05	2.11E+04	8.61%
Titanium (Ti)	2.23E+05	7.19E+04	32.24%

2.4 Preparation of coupons for shipment

Following spore deposition and settling, the acrylic chamber was removed and the coupons were aseptically removed and placed in sterile 16 mL screw cap vials, inoculated side down. The use of this size vial guaranteed that the inoculated side of the coupons could lean diagonally against the vial wall without coming in contact with the bottom, and that only the extreme upper and lower edges of the coupon contacted sides of the vial. Coupons were held in place by a sterile foam plug that was inserted into the mouth of the vial.

Figure 2. Gas control and nebulizer unit

Figure 3. Deposition system base plate with coupons in place

2.5 Transport of coupons to vendor facilities

Coupons in vials were grouped in lots of 25 in zip lock plastic bags and shipped overnight to vendors. In order to determine the fate of spores on the coupons during shipment, several batches of vials were shipped round trip from JPL to vendors to JPL. The coupon assay values for the round trip coupons were compared with base line assay values for that particular coupon run.

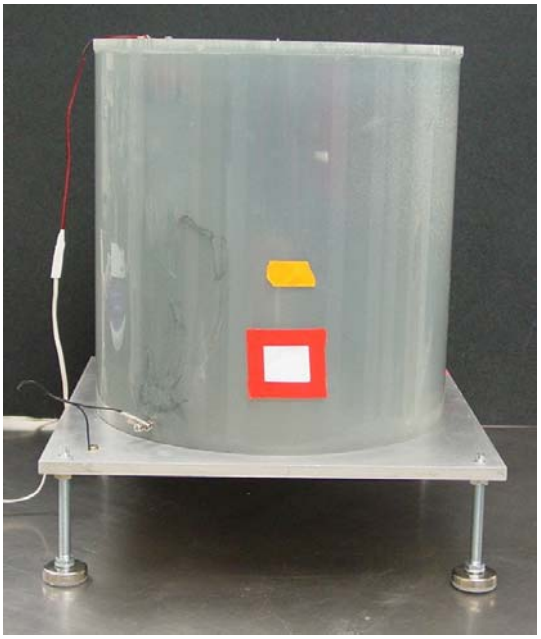

Figure 4. 20-liter deposition chamber on base plate

2.6 Coupon assay technique

A modified NASA standard assay [1], *sans* R2A minimal media plate counting of heterotrophic microflora, was used for microbiological assays. Assays were conducted on all test materials to determine the base line deposition levels (CFU/coupon) prior to shipping and cleaning, after round trip mailing to determine the number of spores lost during transport, and after cleaning procedures. In general, 10 coupons per test material per vendor cleaning process were assayed, although in some instances more coupons were assayed per material.

Four milliliters of sterile water were added to each coupon-containing screw cap vial. Vials were placed on a high-speed vortex mixer for approximately 15 seconds, and then sonicated to remove spores from coupon surfaces. The sonication procedure was accomplished by submersing the vials up to the 4 mL level in the tank of a Branson 8500 Ultrasonication unit for two minutes. Vials were heat shocked at 80^{o}C in a water bath for 15 minutes to kill vegetative cells, and then cooled to 20^{o}C in an ice bath.

Four milliliters from each vial was aseptically transferred to 100 x 15 mm Petri dishes to which ~25 mL of sterile

trypticase soy agar (TSA) was added. After cooling the plates were incubated at 32° C for 72 hours and the resulting bacterial colonies (CFU) were counted.

2.7 Coupon cleaning methods

As stated previously, three vendor-cleaning methods were evaluated in this study. A brief description of each method is provided below.

Vendor A—Inoculated coupons were placed in a stainless steel basket and allowed to soak in a 66° C aqueous cleaning solution (pH 11) for 10-15 minutes, followed by 2-3 minutes of sonication in the same tank. The basket containing the coupons was then rinsed in deionized water and transferred to a second rinse tank. Coupons were dried in the basket by placing the basket in a 71° C hot zone used in conjunction with a nitrogen drying stream.

Vendor B—Deionized water of 18-megohm purity (ultrapure water, UPW) was used as the cleaning agent. Coupons bearing the aerosol deposited bacterial spores were placed in covered beakers containing UPW (1 liter UPW per cubic foot of volume = 3.5E-5 ml per cc of beaker volume). Beakers containing coupons were placed in an ultrasonic bath and sonicated for a minimum of 5 minutes then transferred to a second beaker and the sonication process was repeated. Coupons were removed from the beakers and rinsed with UPW. After the coupons dried, they aseptically transferred to sterile 16 ml screw caped vials and shipped to JPL for microbial assay.

Vendor C—Vendor C's process utilized manipulation of cleaning solution vapor pressure to initiate bubble formation or cavitation on the surface of spore inoculated coupons. Formation and collapse of the bubbles at nucleation sites (surface imperfections or spores) is intended to disrupt the boundary layer on the material surface and dislodge particles. For this procedure, inoculated coupons were placed in a holding rack (ten coupons per material type) and lowered into the processing tank of the cleaning apparatus. The cleaning fluid was heated to 60°C under pressure after which the pressure was cycled (lowered, then raised back to the original level) to initiate cavitation and boundary layer disruption on the coupon surfaces. A standard treatment of 100 pulses each comprised of 1.2 seconds at 1 KHz was used.

A total of four cleaning solvents were used by this vendor:

Process 1—high purity water plus 1.6% hydrogen peroxide (vol/vol) (1 gal 30% H_2O_2 = 0.3 gal H_2O_2; (.3/19)*100 = % H_2O_2)

Process 2—Potassium fluoride (Kyzen proprietary formulation) diluted in high purity water

Process 3—2.6% cold sterilant (22% hydrogen peroxide, 4.5% peracetic acid), 97.4% high purity water; rinse-aqueous 5.3% hydrogen peroxide solution.

Process 4—Ultrapure water with no additives

3. RESULTS/DISCUSSION

When working with bacterial endospore suspensions, there can be a significant degree of variance associated with experimental results. Spores pose numerous problems for standard quantitative analyses. This study represents a major step forward in the deposition of spores on surfaces for cleaning studies. Previous attempts at uniform spore deposition have been fraught with difficulty, often dispersing spores in relatively large droplets, containing numerous spores in aqueous suspension. The deposition chamber fabricated and utilized in this study facilitates the uniform aerosol deposition of single spores onto various spacecraft-qualified coupon surfaces (Fig. 5). An extensive microscopic survey of spore-deposited slides allowed for the rapid identification of refractile spore bodies. Of 70 fields-of-view that underwent post-deposition spore enumeration, all of the fields that contained one or more spores contained only single spores of approximately 1 x 2 microns in size; no clusters of spores were observed. This method appears to effectively deposit spores as single entities. Since bacterial aerosols dispersed into atmospheres of relative humidity of less than 80% are reportedly "instantaneously dry," we presume that under our conditions of approximately 25% relative humidity this is also the case [2].

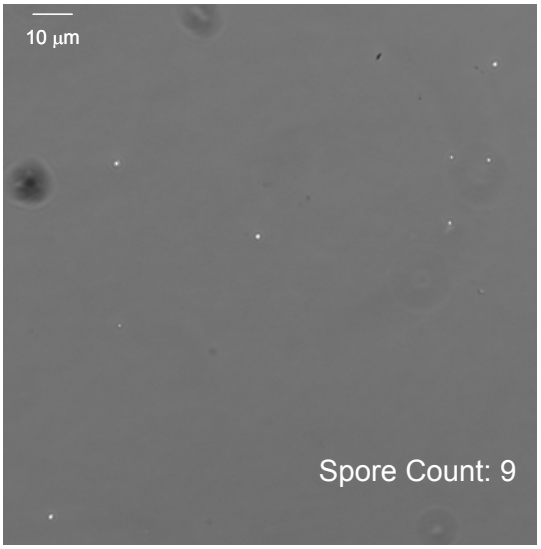

Figure 5. Phase contrast microscopy validation of aerosol deposition of spores

While glass microscope slides were adequate in determining the qualitative nature of the spore deposition method, the

validation and quantitation of spores numbers per given surface area was accomplished via plate-count, culture based techniques as described above. The goal was to deposit 10^5 spores (measured as colony forming units (CFU)) per coupon surface, so that the ability of each cleaning method to deliver a four-log reduction in viable spore burden could then be assessed by CFU enumerations. A sampling of the assay results arising from each subset of spacecraft-material coupons used in this study appears in Table 1. It should be noted that variation per coupon within a given material is comparable to the overall variation associated with inoculation among the different types of coupon surfaces.

With respect to removal of spores from coupon surfaces, it is clearly apparent that some vendors, and some methods for that matter, are far more effective than others. Keeping in mind that the goal of such cleaning efforts is a four-log reduction in viable spore burden, a threshold value of 10 CFU was used to delineate "effectively-sterilizing" and ineffective methods of cleaning to this degree. For a method to be deemed "effectively sterilizing" it must achieve a four-log reduction in spore burden, and thus reduce a coupon's original spore titer of ~ 1 x 10^5 CFU to less than 1 x 10^1 CFU following treatment. Six distinct methods each employed by one of three individual vendors were evaluated in this study. The cleaning approaches enveloped a wide range of effectiveness, as depicted in Table 2. Cleaning by Method A, largely freon degreasing, yielded a mere 2-log reduction in spore burden and was deemed ineffective. This method yielded between 1.9 x 10^2 and 2 x 10^3 CFU following treatment, depending on material tested (Method A). The cleaning results of Method B were a bit more promising, as 6 of the materials tested were cleaned to a level near or below threshold (ranging from 2.3 to 2.2 x 10^1). There remained much room for improvement, however, as the 5 materials whose cleaning did not show a 10^4 fold reduction in bioburden exhibited anywhere from 2.4 x 10^2 to 1 x 10^3 CFU, the latter representative of a mere two-log reduction in spore burden.. Method C actually employed a single general process with four variations (C1-C4) representing distinct cleaning solutions in their attempt to achieve NASA-defined sterility. C1 involved the use of H_2O_2 and achieved a four-log reduction in CFU across the board, with only 3 materials presenting any CFU at all (Kapton, Chemfilm, and Black Epoxy Paint; Method 1).

With the exception of its effectiveness at cleaning stainless steel, C2 was equally impressive, leaving 10 of 11 materials tested "sterile" by the NASA definition. Method 3, potassium fluoride-based cleaning, truly cleaned to sterility, rendering all eleven materials completely devoid of viable endospores (Method 3). Unfortunately for NASA, potassium fluoride is extremely caustic and this method's compatibility with spacecraft hardware and capacity for use in future spacecraft decontamination is, at best, improbable. C2 also showed obvious compatibility issues with several of the materials making it an unlikely candidate as well. C1, H_2O_2 solution, appears to be compatible with all materials except black anodized aluminum where it appears to have oxidized the black pigment.

In order to investigate whether spore particles were being removed (cleaned) by C-4 or simply sterilized (killed in place), the same method was used with pure water.

Unlike cleaning solutions 1, 2, and 3 of Vendor C, cleaning solution 4 (DI H_2O) proved ineffective in removing 10^4 endospores from coupon surfaces, as each of the eleven materials remained burdened above threshold following treatment. Astroquartz was the only material that appeared to have had undergone any considerable spore reduction (Method C-4).

4. CONCLUSIONS

Previous surface cleaning studies have focused on the use of clean-room wipes and the application of standard alcohol/water solutions [3, 4]. These have shown that the very nature of the materials being inoculated is often of larger consequence to the resulting cleanliness than the cleaning solution being applied. In contrast, the results arising from this (Table 2), and a previous study, focusing on cleaning processes that would be used prior to items entering clean-room environments [5], appear to be largely material independent. Further evaluation of the aforementioned cleaning systems is underway as to determine the practicality of a robust system capable of achieving surface cleanliness approaching sterility. This study has effectively demonstrated proof of concept.

If we were to rank or recommend the aforementioned cleaning methods based on their performance in removing bacterial endospores from spacecraft-qualified materials, contenders would fall in line as follows:

#1. Cleaning method C-3

Pros: The most effective cleaning method, cleans all materials to "sterility"

Cons: Caustic nature of KF sterilant, unlikely to be compatible with spacecraft hardware

#2. Cleaning method C-1

Pros: Very effective cleaning method, cleans all materials to "sterility"

Cons: Oxidative nature of H_2O_2 sterilant, possibly detrimental to circuitry and spacecraft hardware

#3. Cleaning method C-2

Pros: Effective cleaning of nearly all materials (exception: stainless steel)

Cons: Causes discoloration of most materials tested

#4. Cleaning method B

Pros: Effective cleaning of 6 of 11 materials tested

Cons: Lackluster performance on 5 of 11 materials tested

The remaining cleaning methods did not perform adequately enough to discuss in any further detail.

In conclusion, this is a work in progress. We consider the aerosol deposition of spores on surface materials an important step towards quantitative evaluating there removal and what can be expected from a given cleaning procedure. Base on the results presented here, the use of hydrogen peroxide with the vacuum cavitation system appears most promising for the reduction of viable spores with minimum obvious change in surface properties of the materials tested. Follow on research should better identify not only which method is "best" from the perspective of cleaning to sterility but may suggest special applications for different procedures.

Table 2. Comparison of the efficacy of six cleaning methods

Material	Cleaning Method						
	Inoculated Count (cfu)	Method A (mean cfu)	Method B (mean cfu)	Method C-1 (mean cfu)	Method C-2 (mean cfu)	Method C-3 (mean cfu)	Method C-4 (mean cfu)
Aluminum, bare (AL)	2.03E+05	TNTC	3.69E+02	0.00E+00	3.40E+00	0	TNTC
Aluminum, black anodized (BA)	1.15E+05	9.61E+02	2.33E+00	0.00E+00	0.00E+00	0	TNTC
Aluminum, black epoxy paint (BP)	1.48E+05	1.01E+03	2.82E+02	2.00E-01	0.00E+00	0	TNTC
Aluminum, chemfilmed (CF)	7.13E+04	1.33E+03	2.42E+02	2.20E+00	0.00E+00	0	TNTC
Aluminum,clear anodized (AC)	9.38E+04	1.78E+03	2.09E+01	0.00E+00	0.00E+00	0	TNTC
Aluminum, white paint (WP)	1.84E+05	8.38E+02	2.25E+01	0.00E+00	0.00E+00	0	TNTC
AstroQuartz (AQ)	1.19E+05	4.12E+02	1.73E+01	0.00E+00	2.20E+00	0	2.35E+02
Graphite composite (GC)	2.03E+05	1.32E+03	6.40E+00	0.00E+00	0.00E+00	0	TNTC
Kapton (K)	3.48E+05	8.48E+02	1.01E+03	2.00E-01	0.00E+00	0	TNTC
Stainless steel (SS)	2.45E+05	1.36E+03	4.76E+02	0.00E+00	1.58E+01	0	1.63E+03
Titanium (Ti)	2.23E+05	1.91E+02	1.57E+01	0.00E+00	0.00E+00	0	TNTC
Total (mean cfu)	1.78E+05	1.10E+03	2.24E+02	2.36E-01	1.94E+00	0	1.80E+03
Total (SD cfu)	7.97E+04	5.39E+02	3.13E+02	6.56E-01	4.73E+00	0	5.30E+02

TNTC: Too Numerous To Count: greater than 2,000 CFU per petri plate

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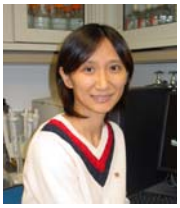
6. BIOGRAPHY

Roger G. Kern, *Ph. D. in Microbiology from U.C. at Davis, is a researcher with specific expertise in the molecular biology, biochemistry and genetics of microorganisms. He is a member of JPL's Biotechnology and Planetary Protection Group. Since arriving at JPL in 1988, he has been task manager on DARPA, DOE sponsored tasks focused on chemoautotrophic growth as it relates to polysaccharide production, and served as molecular biologist associated with two recent flight radiation biology experiments on board STS—42 and 76. Dr. Kern has authored more than 15 papers in bacterial genetics and biochemistry. The primary focus of his research in recent years has been on has been the study of technical issues related to the prevention of forward contamination by spacecraft bound for the surface of Mars. This has included the study of the diversity of microorganisms associated with spacecraft and their assembly environment as well as the evaluation of methods for the killing and removing of microbiological contamination from spacecraft surfaces. An integral part of such study is the development and/or adaptation of methods for the detection of microorganisms and bio-signature molecules such as the one described here.*

Larry E. Kirschner *received his B.S. and M.S. from Abilene Christian University in 1970 and 1976, respectively. After completing his Master's work in microbiology and alternate energy production, he joined the Jet Propulsion Laboratory Planetary Quarantine staff (now Planetary Protection) at the Canaveral Air Force Station, Kennedy Space Center, Florida, for the Voyager project. In addition to developing a large-area sampling method for spacecraft, he was also funded by JPL to work with microbially derived alternate energy systems. The work with microbial systems continued at the General Dynamics/Fort Worth Division where he initiated on-site work for the bioremediation of chlorinated solvents in soil and groundwater. He received the Ph.D. from the University of North Texas in 1993 after conducting research on cometabolic degradation of chlorinated solvents by propane-oxidizing bacteria. At the Dow Chemical/Plaquemine LA, he served as a project lead for research into characterization of wastewater treatment systems. He returned to the Jet Propulsion Laboratory Planetary Protection Group as a Senior Staff member in 2001 and participated in the Mars Exploration Rover and Phoenix Projects, and has contributed to projects to determine the high temperature dry heat resistance of bacterial spores, and the current project, "Cleaning to achieve sterility".*

Myron T. La Duc*. Myron received his B.S. in Bacteriology at the University of Wisconsin-Madison in 1998, and is currently pursuing his Ph.D. in geobiology at the University of Southern California under the advisement of Dr. Ken Nealson. Over the past 7 years Myron has worked at the Jet Propulsion Laboratory as a member of the Center for Life Detection and Biotechnology and Planetary Protection Group. Aside from being a scratch golfer, Myron's extracurricular interests include downhill skiing, surfing, running, and seemingly never-ending "honey do…" lists.*



Fei Chen *received a B.S. in Biochemistry from Beijing University in China and a Ph.D. in Pharmaceutical Sciences from University of Southern California. She was a senior research fellow at California Institute of Technology before she joined the Biotechnology and Planetary Protection Group at the Jet Propulsion*

Laboratory. Her current research is focused on developing and evaluating advanced technologies for cleaning and detection of trace levels of bio-signature molecules and spores on spacecraft materials. These technologies will help to protect life detection research of future mission. Her research interests also include applying cutting-edge molecular techniques to study microbial diversity in spacecraft assembly facilities, and studying the survival and reproduction of microbes under Martian conditions.

Kasthuri Venkateswaran's *25 years of research encompass marine, food, and environmental microbiology. Dr. Venkateswaran has applied his research to better understand the ecological aspects of microbes, while conducting field studies in several extreme environments such as deep sea, spacecraft assembly facility clean rooms as well as the space environment in Earth orbit (International Space Station). Of particular interest are microbe-environment interactions with emphasis on the environmental limits in which organisms can live. The results are used to model microbe-environment interactions with respect to microbial detection, and the technologies to rapidly monitor them without cultivation. The bioinformatics databases generated by Dr. Venkateswaran are extremely useful in the development of biosensors. Further, these models or information in database are extrapolated to what is known about spacecraft surfaces and enclosed habitats in an attempt to determine forward contamination as well as develop countermeasures (develop cleaning and sterilization technologies) to control problematic microbial species. Specifically, his research into the analysis of clean room environments using state-of-the art molecular analysis coupled with nucleic acid and protein-based microarray, will allow accurate interpretation of data and implementation of planetary protection policies of present missions, helping to set standards for future life-detection missions.*

7. ACKNOWLEDGEMENTS

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